Pediatr Surg Int (2014) 30:797–802
DOI 10.1007/s00383-014-3539-1

ORIGINAL ARTICLE

Surgical acquired aganglionosis: myth or reality?

M. J. Bag · T. Sáez · J. Varas · H. Vallejos · D. Meléndez · S. Salas · Y. Quiroga · F. Villagrán · S. Montedonico

Accepted: 18 June 2014 / Published online: 15 July 2014

Abstract
Purpose A number of patients operated on for Hirschsprung disease continue to have constipation and abdominal distension for years after surgery. Some authors have proposed that ischemia during surgery may induce secondary aganglionosis. The aim of the present study was to study the effects of ischemia on the enteric nervous system of sigmoid colon in an animal model.
Methods A surgical model of colonic ischemia was created. 34 adult Sprague–Dawley rats underwent a laparotomy where the marginal arterioles of the sigmoid colon were ligated. After that, a section in the middle segment of the sigmoid colon was performed followed by an anastomosis. The presence of ischemia was assessed by measurement of visible light spectroscopy tissue oximetry and histological examination. Colonic function was assessed by evaluation of stool weight. Rats were killed at 1, 8 and 12 weeks after the operation. 12 rats were sham-operated. Enteric nervous system was evaluated by means of immunohistochemistry with NGFR p75. Quantitative analysis of the number of ganglia and ganglion cells in the myenteric plexus was performed.
Results The surgical model of colonic ischemia significantly decreased tissue oxygenation (pre-surgical = 54.69 ± 7.32 %; post-surgical = 27.37 ± 9.2 %; $p < 0.001$). There was no disturbance in body-weight gaining in experimental groups and daily stool output did not vary after surgery (pre-surgical = 4.24 ± 0.94 g; post-surgical = 3.82 ± 1 g; $p = 0.09$). All experimental groups showed persistent ganglia. However, there was a significant decrease in the number of ganglia in all the experimental groups compared to control (1w: 45.91 ± 7.66; 8w: 44.17 ± 10.56; 12w: 36.17 ± 15.06 vs control: 56.88 ± 8.66; $p < 0.01$). The number of total ganglion cells was significantly reduced only in the experimental group killed at week 12 compared to control (1w: 539 ± 167.58; 8w: 488.58 ± 154.41; 12w: 343.94 ± 161.91 vs control: 513.96 ± 126.97; $p < 0.01$). The rate of ganglion cells per ganglia was significantly higher in the groups killed at week 1 and 8 versus control group (1w: 11.63 ± 2.53; 8w: 11.11 ± 2.56; 12w: 9.34 ± 1.16 vs control: 9.02 ± 1.81; $p < 0.05$).
Conclusion Long-term follow-up after surgically induced colonic ischemia in the rat showed a decreased number of ganglion cells and ganglia. Nevertheless, it did not produce aganglionosis.

Keywords Postoperative aganglionosis · Postoperative persistent obstructive symptoms · Hirschsprung disease · Intestinal ischemia

M. J. Bag · T. Sáez · J. Varas · H. Vallejos · D. Meléndez · S. Salas · Y. Quiroga · F. Villagrán · S. Montedonico (✉)
Departamento de Ciencias Biomédicas, Edificio Bruno Günther, Escuela de Medicina, Universidad de Valparaíso, Hontaneda, 2664 Valparaiso, Chile
e-mail: sandra.montedonico@uv.cl

M. J. Bag · S. Montedonico
Unidad de Cirugía Pediátrica, Escuela de Medicina, Universidad de Valparaíso, Valparaiso, Chile

Background and purpose

Hirschsprungs disease (HD) is a congenital condition characterized by the absence of ganglion cells in the distal bowel, affecting one in 5,000 born children [1]. The treatment consists of surgical resection of the aganglionic segment followed by a pulled through of normally innervated colon. However, a number of patients varying between 6 and 42 % continue to have long-term persistent

obstructive symptoms [2–6]. As proposed by Langer in his diagnostic and therapeutic algorithm these obstructive symptoms may be secondary to: mechanical obstruction, internal sphincter achalasia, intestinal motility disorder, functional megacolon, recurrent or acquired aganglionosis; on his publication he mentions that the later "acquired aganglionosis" remains one of the controversial element of his algorithm [3]. Some authors have also hypothesized that persistent obstructive symptoms may be secondary to postoperative acquired aganglionosis as a consequence of ischemia during surgery [2, 3, 7–11].

The aim of the present study was to investigate the effects of ischemia on the enteric nervous system of sigmoid colon by means of a surgically created animal model.

Methods

Thirty four adult Sprague–Dawley rats weighing 250–400 g were randomly divided into two groups: control and experimental group. The Ethics Committee of the Universidad de Valparaíso approved all the animal experiments under the Manual for Biosecurity, by the Scientific and Technological National Commission (CONICYT, 2008).

Animal model and assessment of colonic ischemia

The animals underwent general anesthesia through an intraperitoneal injection of xylazine hydrochloride (7.5 mg/kg) and ketamine hydrochloride (50 mg/kg).

In the experimental group a 8 cm midline suprapubic skin incision was made, the fascia was reflected, the peritoneum was opened, the intestines were retracted laterally for better exposure and sigmoid colon was identified. After that colonic ischemia was created by ligating the marginal arterioles in a length of 5 cm using prolene 5-0 followed by a section in the middle of the ligated segment and an anastomosis with running suture using prolene 8-0 under a surgical microscope (Mentor CM6X). Bowel humidification was maintained with constant instillation of saline solution. After that, abdominal wall was closed in two layers.

Ischemia was assessed in the colon prior ligation and after ligation and anastomosis, with a 1.5 mm fiberoptic catheter-based visible light spectroscopy oximeter (T-Stat 303 Microvascular Oximeter, Spectros, Portola Valley, Calif, USA). The probe was positioned on the serosal surface and the tissue hemoglobin oxygen saturation was measured for a mean period of 3 min.

On weeks 1, 8 and 12 after surgery rats were anesthetized with the same protocol mentioned above. A laparotomy was performed, adhesions were carefully dissected and a segment of 1 cm above and below the anastomosis was resected. Rats were then killed by an intracardiac injection of potassium chloride solution. Specimens were kept in 4 % paraformaldehyde solution until inclusion in paraffin.

Control group consisted in a laparotomy, blunt manipulation of intestines, oximetry assessment with visible light spectroscopy and collection of sigmoid colon specimens.

Histological sections of sigmoid colon from control and experimental groups were stained with hematoxylin–eosin and were blindly reviewed by an expert pathologist seeking for histological findings of ischemia according to a previously described protocol [12].

Assessment of effect of ischemia on ganglia and ganglion cells

Five millimetre thick histological sections were processed for immunohistochemistry with Anti-p75 Nerve growth factor receptor antibody (NGFR p75) according to the following protocol: paraffin-waxed sections were deparaffinized, rehydrated, and then subjected to antigen retrieval by steam heating for 15 min in pH 6 citrate buffer at 95 °C. Sections were incubated with 3 % hydrogen peroxide for 30 min to remove endogenous peroxidase activity and then washed with PBS 1× three times. After that sections were blocked with Cas-block (30 min) and goat serum (30 min) at room temperature, and then incubated overnight at 4 °C with primary antibody Anti-p75 NGF receptor with a dilution of 1:500 (Rabbit polyclonal, ab8874, Abcam®). Next day, after three consecutive washes with PBS 1×, they were incubated with goat serum anti-rabbit IgG (H+L) (1:500; Invitrogen®) conjugated with peroxidase (60 min). Vector® Nova Red, horseradish-peroxidase (HRP)-chromogen was applied on slides for 30 s. Sections were counterstained in Harris hematoxylin.

At least two sections from each specimen were observed under a light microscope (Olympus® CX21). The number of ganglia in the myenteric plexus in each section as well as the number of cells in each ganglia were counted.

Sections were microphotographed with objectives 10× and 40× (Olympus® CX 81 microscope, digital camera DP71 and software DP Controller, Olympus®.). The area of the section occupied by ganglia was measured using an image processing software (Image-Pro, MediaCybernetic®).

Assessment of functional effect of colonic ischemia

Rat body weight was registered prior to surgery and at the day of sacrifice. Fecal output was assessed 10 days before and 21 days after surgery, by weighing dry feces every 24 h according to a previously described technique [13].

Statistical analyses

Statistical analyses of data were performed with StataSE 12, StataCorp® using non-parametric tests. Differences among measurement in the same rat (tissue oxygenation and deposition weight) were analyzed using Wilcoxon signed-rank test, and differences among groups were assessed using the Mann–Whitney U test. p value <0.05 was considered to be statistically significant.

Results

Induced colonic ischemia assessment

Tissue oxygen saturation of the sigmoid colon before surgery procedure was 55.44 ± 8.69 %. After surgery, tissue oxygen saturation dropped to 25 ± 8.44 % (Table 1).

In the group killed at week 1, there was inflammatory infiltration in all the sections and variable grades of edema and crypts distortion; alterations corresponded to grade 1 and 2 of Chiu classification [12]. Some sections evidenced reparative changes in epithelium (diminished numbers of crypts). There were no significant alterations in the histological observations of experimental groups killed after 8 and 12 weeks.

Enteric nervous system assessment

In the control group, the mean number of ganglia was 56.88 ± 8.66 and the number of ganglion cells per ganglia was 513.96 ± 126.97 (Table 2).

Ganglion cells and ganglia were visible in all experimental groups regardless of the time of sacrifice. No postoperative aganglionosis was found in this study. However, a significant reduction in the number of ganglia was found at 1, 8 and 12 weeks after surgery in the experimental group. Total number of ganglion cells per section was similar to control group at 1 and 8 weeks after surgery but were significantly reduced on postoperative week 12. The number of ganglion cells per ganglia was higher at week 1 and 8 compared to control group; at week 12 the number of ganglion cells per ganglia was similar to control (Table 2; Fig. 1).

Table 1 Bowel ischemia assessment with visible light spectroscopy

	Before bowel surgery (mean ± SD), $n = 22$	After bowel surgery (mean ± SD), $n = 22$	p value
Tissue oxygen saturation	55.44 ± 8.69 %	25 ± 8.44 %	< 0.01

Ganglia area measurement

Ganglia area in the control group was 0.0544 ± 0.0241 mm^2. Ganglia area in all experimental groups was reduced. This reduction was significant on postoperative weeks 1 and 12 (Table 3).

Body weight

There was no variation of body weight between pre and postoperative measurements in the group of rats killed on week 1. Preoperative and postoperative body weight of rats killed on week 8 were 273 ± 17.18 and 315.6 ± 43.49 g, respectively, with and weight gaining of 5.33 ± 4.41 g per week. Preoperative and postoperative body weight of rats killed on week 12 were 293.57 ± 21.06 and 349.14 ± 31.75 g, respectively, with and weight gaining of 6.94 ± 2.32 g per week. No rat showed failure to thrive during the experiment.

Fecal output measurement

We measured 24 h fecal output of rats in the experimental group 10 days before and 21 days after surgery. Preoperative daily fecal output was 4.24 ± 0.94 g. Postoperative daily fecal output was 3.82 ± 1 g. There was no significant change in the overall fecal output ($p = 0.09$).

Discussion

The majority of children operated on for Hirschsprung's disease have a good outcome. However, long-term follow-up

Table 2 Number of ganglia and ganglion cells per section in control and ischemic bowel

	Control (mean ± SD), $n = 12$	1 week after bowel ischemia (mean ± SD), $n = 10$	8 weeks after bowel ischemia (mean ± SD), $n = 5$	12 weeks after bowel ischemia (mean ± SD), $n = 7$
Number of ganglia	56.88 ± 8.66	45.91 ± 7.66*	44.17 ± 10.56*	36.17 ± 15.06*
Number of ganglion cells	513.96 ± 126.97	539 ± 167.58	488.58 ± 154.41	343.94 ± 161.91*
Number of ganglion cells per ganglia	9.02 ± 1.81	11.63 ± 2.53*	11.11 ± 2.56**	9.34 ± 1.16

* $p < 0.01$ versus control group

** $p < 0.05$ versus control group

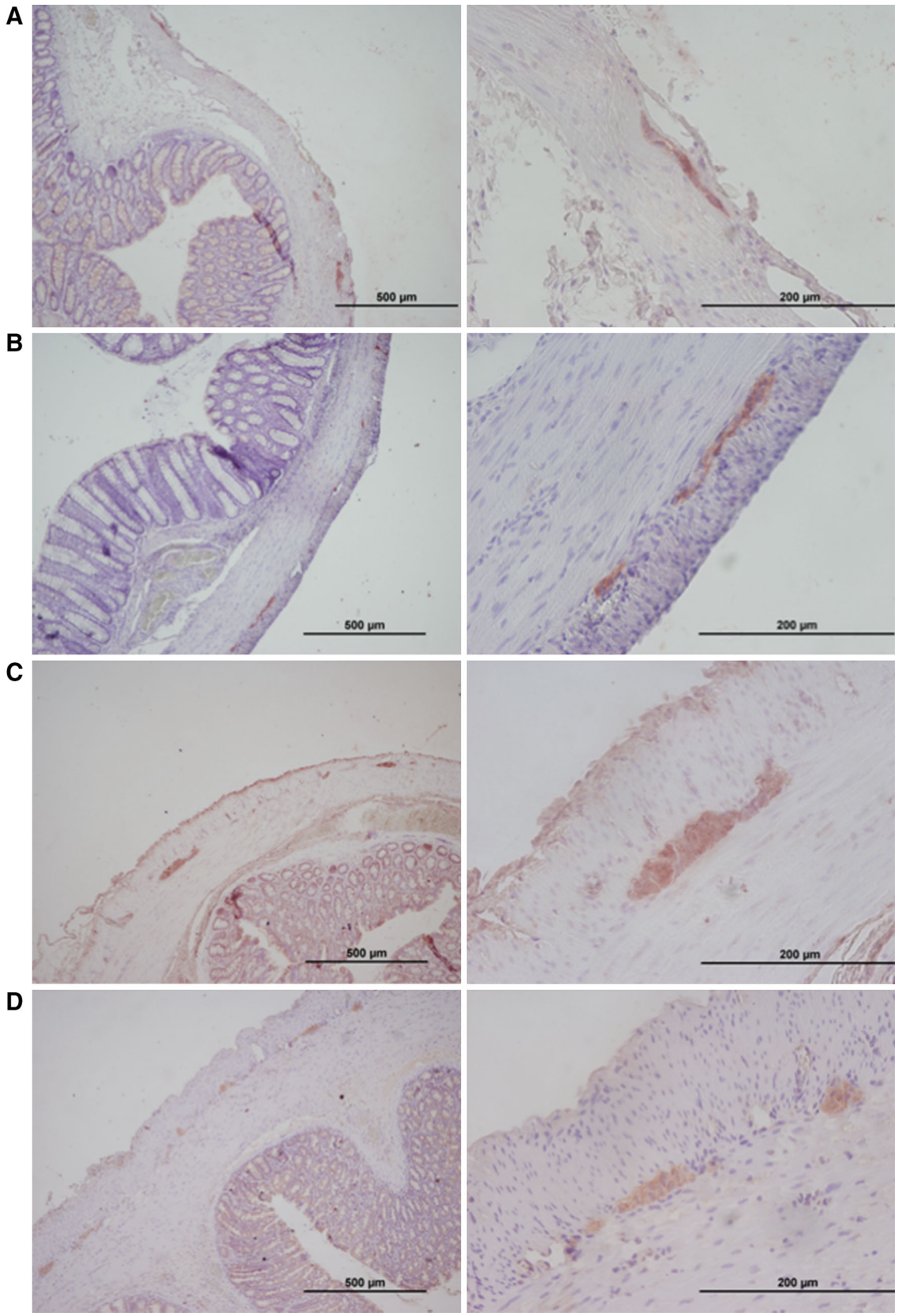

Fig. 1 Ganglion cells in the myenteric plexus of the distal rat colon in control and experimental groups with NGFR p75 immunohistochemistry. **a** Control group. **b** 1 week after bowel ischemia. **c** 8 weeks after bowel ischemia. **d** 12 weeks after bowel ischemia

Table 3 Area of ganglia

	Control (mean ± SD), $n = 12$	1 week after bowel ischemia (mean ± SD), $n = 10$	8 weeks after bowel ischemia (mean ± SD), $n = 5$	12 weeks after bowel ischemia (mean ± SD), $n = 7$
Area of ganglia	0.0544 ± 0.0241 mm^2	0.0308 ± 0.0081 $mm^{2,*}$	0.0399 ± 0.0014 mm^2	0.0299 ± 0.0139 $mm^{2,*}$

* $p < 0.01$ versus control group

shows that a number of patients continue to have significant residual problems. Twenty years ago, Marty et al. [14] reported a 7.5 % incidence of persistent obstructive symptoms after surgery for HD in a large series of patients. Recently, Menezes et al. [15] reported a 30 % incidence of postoperative obstructive symptoms, and Chumpitazi et al. [16] described an incidence up to 88 %. These authors have suggested that previous reports had underestimated the incidence of residual problems after surgery. Postoperative obstructive symptoms may be secondary to surgical complications like anastomotic stricture or a twisted pull-through. They may be also secondary to a residual dysganglionosis or a residual aganglionosis: either a retained aganglionic rectum or an aganglionic bowel pull-through. In 1965, Ehrenpreis [17] described for the first time the presence of postoperative acquired aganglionosis; he hypothesized that vascular disturbance at the time of the first pull-through surgery in a 2-years-old girl may have caused degeneration of ganglion cells in the narrow segment of colon resected 4 years later for obstructive symptoms. Since Ehrenpreis description, postoperative acquired aganglionosis secondary to surgical ischemia has been mentioned in the specialized literature as a possible cause of long-term persistent obstructive symptoms by many authors [2–4, 7–10, 18]. Cohen et al. [8] referred it to be present in about 1.5 % of operated patients. Langer et al. [3] found that 10 of the 49 reoperated children for severe obstructive symptoms had aganglionosis on new rectal biopsies. Review of all the original pull-through specimens showed aganglionosis or hypoganglionosis in only three of them. Moore et al. described a 14 % incidence of persistent obstructive symptoms after pull-through surgery. New rectal biopsies of these patients showed aganglionosis in 29 % and features of intestinal neuronal dysplasia in 64 % of them [19].

Although present in the literature for a long time, most animal models study the effects of ischemia–reperfusion on intestinal motility [20, 21]. However, few experimental studies have focused on the consequences of pure colonic ischemia on the enteric nervous system. Moreover, these studies have shown contradictory results [22, 23].

In the present study, we obtained a model of ischemia by a surgical procedure that involves section of bowel and termino-terminal anastomosis, mimicking the surgical procedure that is performed in patients with HD. In our study, effective postoperative ischemia was demonstrated by means of tissue oximetry, which constitutes a precise and objective measurement, and also by means of histopathology.

In the present study follow-up of histological changes as long as 12 postoperative weeks, about 7 human years in rat age [24, 25], did not show aganglionosis. These findings clearly reflect to what others have previously showed: De Villiers [23] also disproved the theory of ischemia as being causative of colonic aganglionosis in an experimental animal model in dogs.

However, our study evidenced a significant reduction in the number of ganglia in all the experimental groups; supporting this finding, the area occupied by ganglia was decreased in all experimental groups. It was interesting to note that the number of ganglion cells remained similar to control group on experimental weeks 1 and 8. As a consequence, the number of ganglion cells per ganglia was higher in these groups. These results may be explained by an initial loss of small ganglia, containing <3 cells, soon after bowel ischemia.

On long-term follow-up at 12 weeks post bowel ischemia there was not only a reduction in the number of ganglia but also an important reduction in the absolute number of ganglion cells per section. As a consequence of the later, the number of ganglion cells per ganglia rate remained the same.

Ikeda et al. [26] evidenced that the major mode of cell death in rats' small intestinal epithelial cells induced by ischemia–reperfusion was apoptosis. Kondo et al. [27] evidenced neuronal death in rats' brain even 1 year after transient forebrain ischemia–reperfusion injury. Yang et al. [28] observed that the hypoxia-inducible factor (HIF)—a factor that modulates adaptive and protective mechanism, triggered by hypoxia, ischemia, and other patho-physiological conditions—increases in rat hippocampus during chronic ischemia. But the expression of downstream genes, some of which are pro-apoptotic whereas others are pro-survival, are up regulated in the early stage of hypoperfusion, thus the lack of protective effect of HIF. Winerdal et al. [29] also evaluated long lasting effect of hypoxia in central nervous system of rats showing that inflammatory cells are still active even after 3 months of follow-up. We believe that our findings might also be explained by apoptosis of ganglion cells mediated by trophic factors and

modulation cells. These mechanisms may be subject of further studies.

Silva et al. [30] in their study of ganglion cells characteristic in ischemia–reperfusion model, found that there was a significant decrease in the stool output on day 21st after surgery in the group with the longest period of ischemia. However Hukuhara et al. [20] studied selective ischemia in the colon of dog model; they observed histological alteration of all the ganglion cells, however no functional repercussion was evident in up to 350 days. Suzuki et al. [21] investigated the effects of ischemia–reperfusion on intestinal motility and showed that motility alterations were reversible up to the 14th post-surgical day. In our study there was not any significant difference in the stool output after surgery, neither was any disturbance in weight gaining; thus we concluded that there was no functional effect of surgically induced colonic ischemia.

In conclusion, the present study does not support the presence of aganglionosis after surgically induced ischemia on sigmoid colon of rats. There is a reduction of ganglia and ganglion cells in long-term follow-up with no associated motility disorders.

Acknowledgments This study was supported by DIPUV 03/2009, Universidad de Valparaíso, Chile. The authors would like to thank Professor Eva Madrid for the statistical analysis of the results.

References

1. Puri P, Montedonico S (2008) Hirschsprung's disease: clinical features. In: Holschneider AM, Puri P (eds) Hirschsprung's disease and allied disorders, 3rd edn. Springer, Heidelberg, pp 107–113
2. Teitelbaum DH, Coran AG (2006) Hirschsprung's disease and related neuromuscular disorders. In: Grosfeld JL (ed) Pediatric surgery, 6th edn. Mosby Inc Elsevier, Philadelphia, pp 1514–1559
3. Langer JC (2004) Persistent obstructive symptoms after surgery for Hirschsprung's disease: development of a diagnostic and therapeutic algorithm. J Pediatr Surg 39(10):1458–1462
4. Dasgupta R, Langer JC (2008) Evaluation and management of persistent problems after surgery for Hirschsprung disease in a child. J Pediatr Gastroenterol Nutr 46:13–16
5. Little DC, Snyder CL (2008) Early and late complications following operative repair of Hirschsprung's disease. In: Holschneider AM, Puri P (eds) Hirschsprung's disease and allied disorders, 3rd edn. Springer, Heidelberg, pp 375–385
6. Ralls MW, Coran AG, Teitelbaum DH (2012) Reoperative surgery for Hirschsprung disease. Semin Pediatr Surg 21(4):354–363
7. Touloukian RJ, Duncan R (1975) Acquired aganglionic megacolon in a premature infant: report of a case. Pediatrics 56(3):459–462
8. Cohen MC, Moore SW, Neveling U et al (1993) Acquired aganglionosis following surgery for Hirschsprung's disease: a report of five cases during a 33-year experience with pull-through procedures. Histopathology 22(2):163–186
9. Langer JC (2012) Laparoscopic and transanal pull-through for Hirschsprung disease. Semin Pediatr Surg 21(4):283–290
10. Moore SW (2008) Neurocristopathies and particular associations with Hirschsprung's disease. In: Holschneider AM, Puri P (eds) Hirschsprung's disease and allied disorders, 3rd edn. Springer, Heidelberg, pp 253–266
11. Kapur RP (2009) Practical pathology and genetics of Hirschsprung's disease. Semin Pediatr Surg 18(4):212–223
12. Chiu ChJ, McArdle AH, Brown R et al (1970) Intestinal mucosal lesion in low-flow states. Arch Surg 101:478–483
13. Bustorff-Silva J, Perez CA, Atkinson JB et al (1999) Effects of intraabdominally insufflated carbon dioxide and elevated intra-abdominal pressure on postoperative gastrointestinal transit: an experimental study in mice. J Pediatr Surg 34(10):1482–1485
14. Marty TL, Seo T, Matlak ME et al (1995) Gastrointestinal function after surgical correction of Hirschsprung's disease: long-term follow-up in 135 patients. J Pediatr Surg 30(5):655–658
15. Menezes M, Corbally M, Puri (2006) Long-term results of bowel function after treatment for Hirschsprung's disease: a 29-year review. Pediatr Surg Int 22(12):987–990
16. Chumpitazi BP, Nurko S (2011) Defecation disorders in children after surgery for Hirschsprung disease. J Pediatr Gastroenterol Nutr 53(1):75–79
17. Ehrenpreis TH (1965) Acquired megacolon as a complication of recto-sigmoidectomy for Hirschsprung's disease. Arch Dis Childh 40:180–182
18. de la Torre L, Langer JC (2010) Transanal endorectal pull-through for Hirschsprung disease: technique, controversies, pearls, pitfalls, and an organized approach to the management of postoperative obstructive symptoms. Semin Pediatr Surg 19(2):96–106
19. Moore SW, Millar AJW, Cywes S (1994) Long-term clinical, manometric, and histological evaluation of obstructive symptoms in the postoperative Hirschsprung's patient. J Pediatr Surg 29(1):106–111
20. Hukuhara T, Kotani S, Sato G (1961) Effects of destruction of intramural ganglion cells on colon motility: possible genesis of congenital megacolon. Jpn J Physiol 11:635–640
21. Suzuki M, Takahashi A, Toki F et al (2008) The effects of intestinal ischemia on colonic motility in conscious rats. J Gastroenterol 43(10):767–773
22. Martinez-Almoynar C, Claver M, Monereo J (1975) Experimental reproduction of Hirschsprunǵdisease with chronic colonic ischemia. Rev Esp Enferm Apar Dig 46(4):349–364
23. De Villiers DR (1966) Ischemia of the colon: an experimental study. Br J Surg 53(6):497–503
24. Sengupta P (2013) The laboratory rat: relating its age with human's. Int J Prev Med 4(6):624–630
25. Suckow M, Weisbroth S, Franklin C (2006) The laboratory rat. Elsevier Academic Press, London
26. Ikeda H, Suzuki Y, Suzuki M et al (1998) Apoptosis is a major mode of cell death caused by ischaemia and ischaemia/reperfusion injury to the rat intestinal epithelium. Gut 42(4):530–537
27. Kondo Y, Asanuma M, Nishibayashi S et al (1997) Late-onset lipid peroxidation and neuronal cell death following transient forebrain ischemia in rat brain. Brain Res 772(1–2):37–44
28. Yang Y, Zhang J, Liu H et al (2013) Changes in levels of hypoxia-induced mediators in rat hippocampus during chronic cerebral hypoperfusion. Neurochem Res 38(11):2433–2439
29. Winerdal M, Winerdal ME, Kinn J et al (2012) Long lasting local and systemic inflammation after cerebral hypoxic ischemia in newborn mice. PLoS One 7(5):e36422
30. Silva MACP, de Meirelles LR, Bustorff-Silva JM (2007) Changes in intestinal motility and in the myenteric plexus in a rat model of intestinal ischemia-reperfusion. J Pediatr Surg 42(6):1062–1065